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(54) Title: BACTERIAL COMPOSITIONS

(57) Abstract: The present invention relates to a biologically pure culture of *S. salivarius*, antibacterial compositions comprising same and its use in the treatment of otitis media.

BACTERIAL COMPOSITIONS

FIELD OF THE INVENTION

This invention relates to the treatment of otitis media. More particularly, it relates to prophylactic and therapeutic treatment of patients in need of same. Also provided are compositions and organisms useful in these methods of treatment.

BACKGROUND

Acute otitis media (AOM) is the most common bacterial infection in growing children. Causative bacteria identified include *S. pneumoniae, S, pyogenes, M. catarrhalis,* and *H. influenzae*. It is thought that the bacteria infect the middle ear via the eustacean tube, from the nasopharynx. Common approaches to treatment are use of antibiotics or insertion of tympanostomy tubes. The former approach is questionable on the grounds of antibiotic resistance, and weakening of the natural host defence system, i.e. reduction in number of normal desirable bacteria. The latter is costly and carries risks where it is performed under general anaesthesia, and can result in membrane damage. Alternate approaches to treatment have therefore been explored. These include use of normal flora in the airways to control pathogens.

The ability of the normal flora of the upper airways to produce inhibition of growth of potential pathogens *in vitro* has been well described. Most of this inhibitory activity has been attributed to alpha-hemolytic streptococci. It has also been identified that children who are prone to AOM have significantly fewer alpha-hemolytic streptococci in their normal flora, and these are less likely to be inhibitory to AOM pathogens than those from children not prone to AOM.

In a recent clinical trial (1b), inhibitory alpha-hemolytic streptococci (AHS) were sprayed into the noses of children with AOM. The AHS were *S. mitis, S. sanguis*, and *S. oralis*. Treatment resulted in those children having fewer episodes of AOM. It appears that this result is attributable to increasing the proportion of inhibitory bacteria in the normal flora that are competitive. The AHS employed in the nasal spray are also recognised human pathogens. For example, *S. oralis* and *S. sanguis* are implicated in endocarditis. *S. mitis* is implicated in lung infections, tooth decay, and abscesses. Accordingly, it would be desirable to have a bacteriocin producing organism which is non-pathogenic and which can be used to protect against, or treat AOM. *S. salivarius* is a non-pathogenic organism which meets these requirements.

While *S. salivarius* has previously been identified in the oral cavity, the isolation of several bacteriocin producing strains from the nasopharynx is believed to be unique. Moreover, while *S. salivarius* with activity against *S. pyogenes* and *S. pneumoniae* have previously been identified, the strains provided by the applicant exhibit broader spectrum activity, including against the gram-negative bacteria *M. catarrhalis* and *H. influenzae*.

It is therefore an object of this invention to provide *S. salivarius* useful in the prophylactic and/or therapeutic treatment of otitis media, or at least which provide a useful choice over existing approaches.

SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to a biologically pure culture of *S. salivarius* strain No. 30 on deposit at Deutsche Sammlung von Mikroorganism Und Zellkulturen GmbH, Brawnschweig, Germany under Accession No. DSM 14686.

In a further aspect, the present invention provides an antibacterial composition which includes an organism as defined above.

The invention further provides a therapeutic formulation comprising an organism as defined above in combination with a diluent, carrier and/or excipient.

In a still further aspect, the invention provides a composition formulated for respiratory administration, the composition comprising:

(a) one or more salivaricin proteins; and/or

(b) one or more organisms capable of expressing a salivaricin protein;

wherein (a) and (b) are effective against one or more otitis media causing bacteria.

As used herein "respiratory administration" means administration to the upper airways of a patient, including administration intranasally and by inhalation or ingestion through the mouth.

In a yet further aspect, the invention provides a therapeutic composition effective for prophylactic or therapeutic treatment of otitis media in a patient, the composition comprising:

(a) one or more salivaricin proteins; and/or

(b) one or more organisms capable of expressing a salivaricin protein;

in combination with a diluent, carrier and/or excipient.

In one embodiment, the salivaricin protein is a *Salivaricin A*, or *Salivaricin B*.

The invention also provides a prophylactic or therapeutic method of treating a patient to at least inhibit the growth of one or more otitis media causing bacteria comprising the step of administering an effective amount of an organism or composition of the invention to said patient.

Preferably, the bacteria are present in the nasopharynx, or oral cavity.

In a further embodiment, said method includes a preliminary step of pre-treating said patient to at least reduce the bacterial population present in the upper airways, or oral cavity.

Preferably, said pre-treatment comprises the step of administering an antimicrobial or antibiotic, preferably erythromycin, orally to said individual.

In a further embodiment, the invention provides a method of treatment of a patient against otitis media which comprises the steps of:

(a) orally administering to said patient an amount of an antibiotic or antimicrobial effective to reduce the normal microflora including otitis media causing organisms present; and

(b) administering to the resulting bacterially depopulated environment, an organism or *S. salivarius* containing composition of the invention to repopulate said environment.

While the invention is broadly defined as above, those persons skilled in the art will appreciate that it is not limited thereto and that it also includes embodiments of which the following description provides examples.

DESCRIPTION OF THE INVENTION

As outlined above, the present invention provides novel *S. salivarius* strains useful in the treatment or prevention of chronic and acute sinusitis, otitis media, including secretory, chronic and particularly acute otitis media.

Previous investigations by the applicant have located a number of BLIS-producing strains of *S. salivarius* active against certain other bacteria including *S. pyogenes* and *S. pneumoniae*. See, for example, WO 01/27143 which discloses such *S. salivarius* as well as salivaricins A and B produced by same. The term BLIS (bacteriocin-like inhibitory substance) refers to extracellularly released bacterial peptides or proteins that in low concentrations are able to kill certain other closely related bacteria by a mechanism against which the producer cell exhibits a degree of specific immunity.

The previously identified *S. salivarius* were isolated from the oral cavity. *S. salivarius* is not an organism commonly associated with the nasopharynx except in very young children. Nor has BLIS production by *S. salivarius* in the nasopharynx been reported. The applicant has therefore surprisingly now isolated a number of BLIS-producing strains from the nasopharynx. Moreover, these strains exhibit a broad spectrum of activity, including strong inhibitory activity *in vitro* against *S. pyogenes*, *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*, the key causative bacteria in otitis media. Importantly, the new *S. salivarius* strains are also non-pathogenic and are generally recognised as non-haemolytic. In comparison with alpha-hemolytic streptococci such as *S. oralis*, *S. sanguis*, and *S. mitis*, this makes the new strains useful for introduction into the nasopharynx or oral cavity of a patient for protection against otitis media or sinusitis.

A preferred strain No. 30 has been deposited at Deutsche Sammlung von Mikroorganism Und Zellkulturen GmbH, Brawnschweig, Germany under Accession No. DSM 14686 on 14 December 2001.

Usually, this strain has genes encoding both a *Salivaricin A* and *Salivaricin B* and is strongly inhibitory of nasopharyngeal streptococcus, as well as *M. catarrhalis* and *H. influenzae*. Two of three control subjects (but no test subjects with recurrent otitis media) had this strain in the nasopharynx, but not on the tongue. This is contrary to customary observation that *S. salivarius* strains preferably localise on the tongue.

The *S. salivarius* are useful in the treatment of otitis media. To this end, the organisms may be formulated in a composition or therapeutic formulation which further includes a diluent, carrier and/or excipient. It will be appreciated in this context that the term "therapeutic" includes prophylactic formulations.

The compositions and formulations are suitable for use in the treatment or prevention of microbial infections, caused by streptococcus, moraxella, and hemophilus organisms. The compositions and formulations are particularly suitable for use against *S. pyogenes*, *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. Examples of compositions and formulations in which the organisms can be employed include orally administrable medicaments such as capsules, lozenges, syrups and gargles, and topically administrable formulations such as creams and cosmetics and respiratorally administrable medicaments. As noted above, this encompasses administration intranasally and by inhalation through the mouth. For example, nasal sprays, and nebuliser formulations. One currently preferred form is a chewable or suckable lozenge.

The invention also provides further compositions formulated for oral or respiratory administration which comprise one or more salivaricin proteins, and/or one or more organisms capable of expressing a salivaricin protein. The proteins and organisms are effective against one or more otitis media causing bacteria. These respiratorally or orally formulated compositions are founded on the applicants surprising finding that they are active against otitis media causing organisms in the nasopharynx or oral cavity. Such compositions have not previously been produced.

Where *S. salivarius* are being administered, they are preferably provided in freeze dried form. The cells may be reconstituted in liquids such as water and saline. The composition will contain between about 1×10^4 and 1×10^{10} colony forming units (CFU) per ml, preferably about 5×10^8 CFU/ml.

To facilitate administration, these compositions will also be formulated together with an acceptable carrier. The selection of the carrier will be dependent upon the formulation and mode of dispensing involved, but will in any case be a matter of routine choice for the skilled worker in this field. For example, for an oral formulation suitable carriers may be isomalt or skim milk. Tableting aids, excipients, flavourings, and colourings may also be employed as appropriate. The presently preferred orally administerable formulations are blends of freeze-dried *S. salivarius* with skim milk powder.

Where administration using a nebuliser is proposed, the formulation may also include a propellant. Powders, solutions, and suspensions are also possible. Optional but preferred components include non-toxic detergents or surfactants, for example, a Polysorbate.

Also provided by the invention is a method for at least inhibiting the growth of one or more otitis media causing bacteria as identified above. Treatment may be carried out by administering an effective amount of an organism or composition of the invention to a patient in need thereof.

Treatment may be prophylactic or therapeutic. Therapeutic treatment encompasses preventing or reducing the severity of, or associated with the symptoms of otitis media. Prophylactic treatment means treatment of a non-sufferer to prevent or at least reduce the likelihood of that individual suffering from otitis media or associated symptoms. Usually treatment will be effected by administering the organisms, compositions, and formulations to the bacteria present in the upper respiratory tract, or oral cavity. Treatment may be repeated as required. For example, lozenges may be used three or four times a day, or as little as once a year as the need arises.

The new *S. salivarius* strains can also be used as alternatives to known *S. salivarius* strains in the inhibition of other bacteria on which they are known to be effective. See, for example, WO 01/27143 entitled "Lantibiotic". The new organisms may similarly be employed in the compositions and formulations identified in that specification.

In a further embodiment, the methods of treatment may include a step of pre-treating the patient to at least reduce the population of normal microflora including the bacteria to be inhibited, for example in the upper airways or oral cavity. Pre-treatment may be effected by antibiotic

administration. An example of a suitable antimicrobial is clorhexidine. Known antibiotics such as erythromycin, amoxycillin and phenoxymethyl penicillin may be used, particularly for oral administration to the patient.

Various aspects of the invention will now be illustrated in non-limiting ways by reference to the following examples.

EXAMPLES

Example 1

The present example compares the BLIS activities against potential AOM pathogens of streptococcal isolates from the nasopharyngeal and oral microfloras of children who do or do not have recurrent AOM.

Materials and Methods

Patients

Thirty-five children were enrolled in the study, twenty in the recurrent AOM group and fifteen controls. They were all between the ages of twelve months and six years. Children were included in the recurrent AOM group if they had been treated for six or more episodes of AOM in the previous year. Control children had experienced one or fewer episodes of AOM. Children in either group were excluded from the study if they had any of the following: 1) taken antibiotics in the preceding two weeks, 2) previous surgery to their upper airway, including adenotonsillectomy, 3) anatomical abnormalities of their upper airway or, 4) known immune deficiency. Consent was obtained from a parent of each child in accordance with the requirements of the Otago Ethics Committee, who approved the study.

Sampling technique

The children were sampled while under general anaesthetic for either surgical or dental procedures. A nasopharyngeal swab was taken from each nostril using a sterile calcium alginate swab. A catheter enclosing the swab was passed through the anterior nares to minimise contamination. The tip of the swab was then pushed through the end of the catheter and rubbed

against the posterior wall of the nasopharynx. Before the catheter was removed from the nose, the tip of the swab was retracted. The catheter tip was then cut off with sterile scissors before re-exposing the swab and plating the microflora sample directly onto the appropriate agar media. A tongue swab was taken at the time to provide a sample for analysis of the subject's oral streptococcal population. All of the freshly inoculated agar plates were transported to the lab for incubation within two hours.

Microbiology

The nasopharyngeal specimens were plated directly onto (i) Mitis Salivarius agar (Difco Laboratories), a selective medium for streptococci and (ii) chocolate agar (Columbia Agar Base (GIBCO) plus 5% human blood, heated to lyse the erythrocytes prior to pouring into petri dishes) to provide a "total microflora" population. The cultures were grown anaerobically at 35°C for 18 hours. From each Mitis Salivarius agar culture, up to five morphologically distinctive colonies were selected and plated onto blood agar (Columbia Agar Base (GIBCO) plus 5% human blood). These cultures were grown overnight at 37°C in a 5% CO_2 in air atmosphere. The tongue swabbings were plated directly onto Mitis Salivarius agar, grown anaerobically at 35°C for 18 hours and representative colonies tested for BLIS production. Samples of the total nasopharyngeal and tongue microflora cultures were resuspended in sterile skim milk and snap frozen at -20°C prior to storage at –70°C.

Testing for BLIS production

Each of the subcultures of the morphologically-distinct colonies from the Mitis Salivarius agar nasopharyngeal cultures was evaluated for BLIS production using a deferred antagonism test on Trypticase Soy Yeast Extract Calcium Agar (TSYCa) {Trypticase Soy Broth (Baltimore Biological Laboratories, Becton Dickinson and Company, USA) + 2% Yeast extract (Difco Laboratories, Detroit, Michigan, USA) + 1.5% Davis agar (Davis Gelatine Ltd, Christchurch, New Zealand) + 0.1% $CaCO_3$. In addition, the mixed bacterial populations recovered from the tongue and the nasopharygeal swabbings were also tested by this method as a screen for BLIS activity. The deferred antagonism method has been described previously.[2] It involves first incubating a 1-cm wide diametric strip of the test bacteria anaerobically at 35°C for 18 hours on the test agar plate. The streak culture is then scraped from the surface of the medium and the agar

surface is sterilised by inversion for thirty minutes over a chloroform-infused cloth. The agar surface is then exposed to the air to remove residual chloroform, following which 18 hour 35°C Todd Hewitt broth cultures of the bacterial strains to be tested for BLIS sensitivity are streaked across the agar at right angles to the test streak. The plate is then returned to the incubator and after eighteen hours is examined to determine the degree of inhibition of the indicator organisms. For the purposes of the present study bacterial inhibition was considered significant if the zone of inhibition of the indicator streak growth was at least twice the width of the original test streak. Isolates significantly inhibiting the growth of either one or both of the two representative strains of each species of AOM pathogens were considered to be inhibitory to that species.

The indicator strains used as representative of species commonly associated with AOM were *Streptococcus pneumoniae* PK2 and PK34 , *S. pyogenes* FF22 and 71-698, *Moraxella catarrhalis* 4 and 22, and *Haemophilus influenzae* 30 and 33. The *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* indicator strains were clinical isolates taken from the middle ear of children with AOM. The *S. pyogenes* strains were reference isolates used commonly in this laboratory as indicators of streptococcal BLIS.[2] In addition, the inhibitory isolates were tested to determine their BLIS P-type activity against a set of nine standard indicator strains (I1-I9) to enable comparison of their inhibitory activities with those of previously-studied BLIS-positive streptococci.[2]

Six nasopharyngeal streptococcal isolates that showed significant inhibitory activity against at least three of the species of AOM pathogens were each tested by PCR for the presence of structural genes previously shown to encode bacteriocin production in the oral streptococcal species *S. salivarius* (salivaricin A[3] and salivaricin B[4]) and *S. pyogenes* (streptococcin A-FF22[5] and streptin[6]). The procedures for DNA extraction and PCR analysis were as described previously.[3] The (5′-3′) primer pair sequences used were as follows:

Salivaricin A (SalA): SrtAfwd AAGACTTTGATCTCGATTTGAA; SrtArev AAACTAATTTCCAACAAGAACCA

Salivaricin B (SalB): SalBfwd GTGAATTCTCTTCAAGAATTGACTCTT; SalBrev AAAATATTCATACCGCTCTTCC

Streptococcin A-FF22 (Scn): ScnAfwd GCACCTATCCTTCTGAAGAAAG; ScnArev GCACCTAGGCACATTTTTTCTTCC

Streptin (Srt): SrtAfwd AAGACTTTGATCTCGATTTGAA; SrtArev AAACTAATTTCCAACAAGAACCA

DNA extracts from the mixed microfloras from the Mitis Salivarius tongue cultures were also tested by PCR using the salivaricin A and salivaricin B primers as a means of detecting the presence of small numbers of salivaricin-producers in the oral microbiota.

BLIS-producing streptococcal isolates were characterised initially on the basis of their colony appearance on Mitis Salivarius and blood agar media and on their biochemical profiles (API 20 Strep, BioMerieux, France).

Results

Twenty-three (65%) of the AOM-prone and control children sampled had bacterial strains isolated on Mitis-Salivarius agar from their nasopharynx that were inhibitory to AOM pathogens in the deferred antagonism test on TSYCa. The numbers of children in each group with isolates inhibitory to each of the pathogens is listed in Table 1 below. The inhibitory isolates were found in children from both groups and there was no significant difference between the groups with respect to the total number of inhibitory organisms isolated.

Table 1: Number of subjects having streptococcal isolates inhibitory to strains of AOM pathogens in deferred antagonism tests on TSYCa

AOM species	Subjects having isolates inhibitory to indicator strains of the stated species of AOM pathogens		
	Recurrent AOM (%) (N=20)	Controls (%) (N=15)	p-value
Streptococcus pneumoniae	5 (25)	2 (13)	0.67
Streptococcus pyogenes	5 (25)	2 (13)	0.67
Moraxella catarrhalis	10 (50)	7 (47)	0.88
Haemophilus influenzae	2 (10)	2 (13)	0.81

Streptococcal isolates from six children (3 controls and 3 AOM group) were inhibitory to both representative strains of at least three of the species of OM pathogens (Table 2 below). Of these six inhibitory isolates, two were *S. pneumoniae*, one was *S. pyogenes* (*emm*-type 11) and 3 were *S. salivarius*. Both direct culture and PCR analysis of the extracted DNA failed to demonstrate the presence of BLIS-producing *S. pyogenes* or *S. pneumoniae* in the total tongue microflora samples from subjects 25, 38 or 40. *S. pyogenes* strain 38 was shown by P-typing (and confirmed by PCR amplification of the appropriate structural gene) to produce the bacteriocin streptin. Neither of the BLIS-positive *S. pneumoniae* were PCR-positive for any of the tested streptococcal bacteriocin structural genes. Although *salA* was amplified from the tongue microflora of subject 44, no corresponding salivaricin A–producing bacterium could be recovered in culture from that subject's tongue swab. A strong BLIS-producing *S. salivarius* nasopharyngeal isolate that was PCR-positive for both *salA* and *salB* was obtained from subject 30 (in the control group). However, in this subject the same inhibitory *S. salivarius* could not be detected in the corresponding tongue sample by either direct culture or PCR. An apparently unrelated population of *salA*-positive *salB*-negative *S. salivarius* was detected both by PCR and by direct culture in the tongue sample from subject 30. *S. salivarius* strain 44 had inhibitory activity consistent with production of the bacteriocin salivaricin A, and by PCR was positive for the appropriate structural gene, *salA*. What appeared to be the same strain of *S. salivarius* was detected by PCR and was also directly isolated in culture from the tongue flora of subject 44.

Table 2: Detection by PCR of salivaricin A and salivaricin B structural genes in strongly-inhibitory nasopharyngeal streptococcal isolates and in samples of the tongue microflora from these subjects

Inhibitory strain	PCR detection of salivaricin A in		PCR detection of salivaricin B in	
	Inhibitory strain	Tongue microflora	Inhibitory strain	Tongue microflora
Streptococcus pneumoniae 25$^{\phi}$	-	-	-	-
Streptococcus salivarius 30*	+	+	+	-
Streptococcus pyogenes 38$^{\phi}$	-	-	-	-
Streptococcus pneumoniae 40*	-	+	-	-
Streptococcus salivarius 44 $^{\phi}$	+	+	-	-

*From control subjects $^{\phi}$From recurrent AOM subjects

Discussion

The finding of bacteria in the nasopharynx of children that are inhibitory to AOM pathogens *in vitro* is common. The present study however has demonstrated that *S. salivarius* is present in the nasopharynx in some children, and that some strains produce BLIS that is strongly inhibitory *in vitro* to AOM pathogens.

In several previous studies it has been found that there are significant differences between the numbers of organisms displaying in vitro inhibitory activity in nasopharyngeal samples from children prone to AOM and control subjects [7,8,9]However, in none of these studies was the nature of this in vitro inhibition established.

Bernstein found that the predominant AHS in the nasopharyngeal flora of children were *S. mitis* and *S. sanguis*[10]. It is generally known that the non haemolytic streptococcus species, *S. salivarius*, is predominantly present in the oral cavity of humans, and that it is found in highest levels on the tongue. However, a unique finding in the present study was that two children not disposed to AOM harboured strongly inhibitory *Streptococcus salivarius* in their nasopharynxes, without apparently having these same strains on their tongues. The nasopharyngeal isolates of *S. salivarius* from these two children were positive for both the salivaricin A and salivaricin B structural genes. Interestingly, our previous studies have found that *S. salivarius* positive for both of these salivaricins are much less commonly detected in the oral cavity than are *S. salivarius* that are positive only for salivaricin A. Both salivaricin A/salivaricin B positive nasopharyngeal *S. salivarius* isolates from the control subjects had strong *in vitro* inhibitory activity against all tested strains of the AOM pathogens, *S. pyogenes*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. It was of further interest that although inhibitory *S. salivarius* were also recovered from both the nasopharynx and tongue of one child prone to AOM, these *S. salivarius* isolates were positive for salivaricin A, but not for salivaricin B. Taken together these findings are supportive of the novel hypothesis that *S. salivarius* strains positive for both type A and B salivaricins may become established relatively more readily in the nasopharyngeal microbiota than in the oral cavity when compared with *S. salivarius* that are positive only for type A salivaricin. Moreover, *S. salivarius* that are positive for both salivaricins appear to be relatively

more commonly isolated from the nasopharyngeal microbiotas of children who are not prone to develop AOM than from children who appear susceptible to this disease.

BLIS production by *S. salivarius* strains isolated from the oral cavity has been well documented [11,12] and some of these strains are strongly inhibitory to the growth of *S. pyogenes*. However, strain 30 isolated from nasopharyngeal specimens has inhibitory activity not only against *S. pyogenes*, but also against a range of other pathogens including the gram negative *M. catarrhalis* and *H. influenzae*. Since *S. salivarius* is considered to be essentially non-pathogenic it may be an excellent candidate for introduction into the normal nasopharyngeal flora as protection against recurrent AOM.

Example 2

Identification

Strain 30 was isolated from the nasopharynx of a human subject. It grows on Mitis salivarius agar at 37°C, 5% CO_2 with morphology typical of *S. salivarius* as follows:

Colony shape and size: round, 1-2 mm in diameter
Margin (edge): entire (smooth)
Elevation: convex
Colour: blue
Texture: mucoid

On Blood agar [Columbia Agar Base (GIBCO) with 5% human blood] at 37°C, 5% CO_2 in air it is not haemolytic, and exhibits the following morphology:

Colony shape and size: round , <1 mm in diameter
Margin (edge): entire (smooth)
Elevation: convex
Colour: white
Texture: mucoid

The API 20 Strep Identification code for the strain is 5070450, which corresponds to *Streptococcus salivarius* (98.4% identity).

16s rRNA Sequence Analysis with reference to the GENEBANK database established the strain to be *Streptococcus salivarius* (99.9% homology).

Biochemical Characterization

Biochemical characterization of *S. salivarius* 30 was conducted using the API 20 Strep kit (bioMérieux) and API 50 CH (bioMérieux) to investigate carbohydrate metabolism.

The API 20 Strep results are as follows:

Acetone production	positive
Hydrolysis	negative
ß-glucosidase	positive
Pyrrolidonyl arylamidase	negative
a-galactosidase	negative
ß-gluuronidase	negative
ß-galactosidase	positive
alkaline phosphatase	positive
Leucine arylamidase	positive
Arginine dihyrolase	negative
Ribose	negative
L-arabinose	negative
Mannitol	negative
Sorbitol	negative
Lactose	positive
Trehalose	positive
Inulin	negative
Raffinose	positive
Starch	negative
Glycogen	negative
ß-haemolytic	negative

The API 50 CH results are as follows:

Glycerol	negative
Erythritol	negative
D-Arabinose	negative
L-Arabinose	negative
Ribose	negative
D-Xylose	negative
L-Xylose	negative
Adonitol	negative
ß-Methyl-xyloside	negative
Galactose	positive

D-Glucose	positive
D-Fructose	positive
D-Mannose	positive
L-Sorbose	negative
Rhamnose	negative
Dulcitol	negative
Inositol	negative
Mannitol	negative
Sorbitol	negative
a Methyl-D-mannoside	negative
a Methyl-D-glucoside	negative
N-Acetyl glucosamine	positive
Amygdaline	negative
Arbutin	positive
Esculin	positive
Salicin	positive
Cellobiose	positive
Maltose	positive
Lactose	positive
Melibiose	negative
Saccharose	positive
Trehalose	positive
Inulin	negative
Melezitose	negative
D-Raffinose	positive
Amidon	negative
Glycogen	negative
Xylitol	negative
ß Gentiobiose	negative
D-Turanose	negative
D-Lyxose	negative
D-Tagatose	negative
D-Fucose	negative
L-Fucose	negative
D-Arabitol	negative
L-Arabitol	negative
Gluconate	negative
2 ceto-gluconate	negative
5 ceto-gluconate	negative

Inhibitory Activity

Deferred Antagonism test for BLIS Activity

P-type of *S. salivarius* 30

Producer typing (P-type) describes the antimicrobial activity of bacteria against a set of standard indicators. The procedure was first described by Tagg and Bannister (J. Med. Microbiol. 1979; 12: 397-411).

For P-typing *S. salivarius* 30 was grown as a diametric streak culture on a Blood agar + 0.1% calcium carbonate plate or Trypticase soy-yeast extract-calcium carbonate agar (Trypticase soy broth, 30; yeast extract, 20 g; calcium carbonate, 2.5 g; agar, 15 g; distilled water, 1000 ml), incubated at 37°C, 5% CO_2 for 18 h. The growth was then removed and the surface of the plate sterilised with chloroform. Nine indicator strains were then cross-inoculated. After incubation at 37°C, 5% CO_2 in air for 18 h inhibition of growth was recorded. The inhibition patterns were recorded in a code form by considering the nine indicators as three triplets (eg, I1, I2, I3, I4, I5, I6; I7, I8, I9). Positive reactions against each indicator were given a score of 4, 2 or 1 depending on whether the indicator was, respectively, the first, second or third member of the triplet. No inhibition was recorded as zero. The total score of each triplet thus specified uniquely the reactions against the three indicators. The complete P-type code is written as a sequence of three numbers, consecutively defining the reactions within the three triplets.

S. salivarius 30 has a 230 P-type on Blood agar + calcium carbonate, and a 360 P-type on Trypticase soy-yeast extract-calcium carbonate agar when incubated at 37°C, 5% CO_2 in air. However, *S. salivarius* 30 has a 000 P-type on Blood agar + calcium carbonate, and a 777 P-type and Trypticase soy-yeast extract-calcium carbonate agar when incubated at 37°C, anaerobically.

Table 3: Deferred antagonism assay on BACa and TSYECa plates

Incubation Conditions	I1	I2	I3	I4	I5	I6	I7	I8	I9	P-type
BACa –CO_2	-	+	-	+	-	+	-	-	-	250
AnO_2	-	-	-	-	-	-	-	-	-	000
TSYECa –										
CO_2	(-)	2+	+	2+	-	2+	-	-	-	350
AnO_2	4+	4+	3+	3+	3+	2+	3+	3+	2+	777

Deferred Antagonism test for BLIS Activity against Otitis media pathogens

The BLIS activity of *S. salivarius* 30 against a set of Otitis Media pathogens was carried out using the same procedure that was first described by Tagg and Bannister (J. Med. Microbiol. 1979; 12: 397-411), except the indicator strains were as follows: OM1 *Streptococcus pneumoniae*

PK2; OM2 *S. pneumoniae* PK34; OM3 *Streptococcus pyogenes* strain FF22; OM4 *S. pyogenes* strain 71-698; OM5 *Moraxella catarrhalis* 4; OM6 *M. catarrhalis* 22; OM7 *Haemophilus influenzae* 30; OM8 *H. influenzae* 33; OM9 *Micrococcus luteus* T18.

S. salivarius 30 has only activity against one *S. pyogenes* indicator when grown on BACa, while has activity against all indicators when grown on TSYE agar (Table 4).

Table 4: Activity of *S. salivarius* 30 against Otitis media pathogens

Incubation Conditions	OM1	OM2	OM3	OM4	OM5	OM6	OM7	OM8	OM9	OM-type
BACa –										
CO_2	(-)	(-)	+	-	-	-	-	-	-	100
AnO_2	-	-	+	-	-	-	-	-	-	100
TSYE –										
CO_2	+	+	+	+	3+	2+	+	+	3+	777
AnO_2	2+	2+	2+	2+	2+	2+	+	+	2+	777

INDUSTRIAL APPLICATION

The results above demonstrate the inhibitory effect of BLIS production producing *S. salivarius* against streptococcus, moraxella, and haemophilial infections. *S. salivarius* or other organisms producing salivaricins are therefore applicable in methods of prophylactically or therapeutically treating individuals for such infections, including otitis media.

Presently preferred compositions for use in the methods are formulated for respiratory or oral administration. The compositions comprise freeze dried *S. salivarius* BLIS producing strains with a respiratorially acceptable adjuvant, or are formatted as lozenges.

Those persons skilled in the art will understand that the above description is provided by way of illustration only and that the invention is not limited thereto.

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CLAIMS:

1. A biologically pure culture of *S. salivarius* strain No. 30 on deposit at Deutsche Sammlung von Mikroorganism Und Zellkulturen GmbH, Brawnschweig, Germany under Accession No. DSM 14686.

2. An antibacterial composition which includes an organism as defined in claim 1.

3. A therapeutic formulation comprising an organism as defined in claim 1 in combination with a diluent, carrier and/or excipient.

4. A composition formulated for respiratory administration, the composition comprising:

   (a) one or more salivaricin proteins; and/or
   (b) one or more organisms capable of expressing a salivaricin protein;

   wherein (a) and (b) are effective against one or more otitis media causing bacteria.

5. A composition as claimed in claim 4, wherein said otitis media causing bacteria are selected from one or more of the group comprising *S. pyogenes*, *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*.

6. A therapeutic composition effective for prophylactic or therapeutic treatment of otitis media in a patient, the composition comprising:

   (a) one or more salivaricin proteins; and/or
   (b) one or more organisms capable of expressing a salivaricin protein;

   in combination with a diluent, carrier and/or excipient.

7. A composition as claimed in any one of claims 4 to 6, wherein the salivaricin protein is a *Salivaricin A*, or *Salivaricin B*.

8. A composition or formulation as claimed in any one of claims 2, 3 and 5 to 7, formulated for oral, topical, or respiratory administration.

9. A composition or formulation as claimed in claim 8, formulated for oral administration and comprising a chewable or suckable lozenge.

10. A composition or formulation as claimed in any one of claims 2 to 9 comprising *S. salivarius* in freeze dried form.

11. A composition or formulation as claimed in claim 10, wherein the freeze dried *S. salivarius* cells are reconstituted in a liquid selected from water and saline.

12. A composition or formulation as claimed in claim 11 comprising between about 1×10^4 and 1×10^{10} colony forming units (CFU) per ml, preferably about 5×10^8 CFU/ml.

13. A composition or formulation as claimed in any one of claims 10 to 12, further comprising an acceptable carrier.

14. A composition or formulation as claimed in claim 13, further comprising one or more tableting aids, excipients, flavourings, and/or colourings.

15. A composition or formulation as claimed in any one of claims 10 to 13 comprising an orally administerable formulation of a blend of freeze-dried *S. salivarius* and skim milk powder.

16. A composition or formulation as claimed in any one of claims 2 to 8, formulated for respiratory administration as a powder, solution or suspension.

17. A composition or formulation as claimed in claim 16, formulated for use in a nebuliser and further comprising a propellant.

18. A composition or formulation as claimed in claim 16 or claim 17, further comprising a non-toxic detergent or surfactant such as a Polysorbate.

19. A prophylactic or therapeutic method of treating a patient to at least inhibit the growth of one or more otitis media causing bacteria, comprising the step of administering an effective amount of an organism as claimed in claim 1 or a composition as claimed in any one of claims 2 to 18 to said patient.

20. A method as claimed in claim 19, wherein the bacteria are present in the nasopharynx, or oral cavity.

21. A method as claimed in claim 19, wherein said method includes a preliminary step of pre-treating said patient to at least reduce the bacterial population present in the upper airways, or oral cavity.

22. A method as claimed in claim 21, wherein said pre-treatment comprises the step of administering one or more antimicrobials or antibiotics orally to said individual.

23. A method as claimed in claim 19, wherein said antimicrobial or antibiotic is selected from the group comprising chlorexidine, erythromycin, amoxycillin and phenoxymethyl penicillin.

24. A method as claimed in claim 23, wherein said antibiotic is erythromycin.

25. A method of treatment of a patient against otitis media which comprises the steps of:

(a) orally administering to said patient an amount of an antibiotic or antimicrobial effective to reduce the normal microflora including otitis media causing organisms present; and

(b) administering to the resulting bacterially depopulated environment, an organism or *S. salivarius* containing composition of the invention to repopulate said environment.

26. A use of *S. salivarius* strain No. 30 as defined in claim 1, in the manufacture of a medicament for the treatment or prevention of chronic and acute sinusitis, otitis media, including secretory, chronic and acute otitis media.

SEQUENCE LISTING

<110> BLIS TECHNOLOGIES LIMITED

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2004/000025

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 1/20, A61K 35/74, A61P 11/04, A61P 31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABESES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, MEDLINE, CA: Salivarius, salivaricin, streptococcus, otitis, AOM, antibacterial, antimicrobial, respiratory, treatment.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Fujimori, I. et al. 1992. Significance of normal oral flora, particularly group oral streptococci as defense mechanism against infection in health individuals (normal defense mechanism by oral *streptococcus* group). Journal of Japanese Association for infectous Diseases, 66(12), pages: 1634-38. (See abstract).	4-20
X	US 3925160 A (W.Eugene Sanders et al). 9 December 1975. (See whole document)	4-20
X	WO 2001/027143 A1 (University of Otago et al). 19 April 2001. (See abstract and pages 1-5, 10-11, and 16)	4-25

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the prinelple or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
26 May 2004

Date of mailing of the international search report
- 3 JUN 2004

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2004/000025

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Upton, M. et al. 2001. Intra-and interspecies signaling between *Strepococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalAI lantibiotic peptides. Journal of Bacteriology, Vol: 183, No: 13 pages 3931-38. (See abstract and page 3931).	4-20
X	Dempster, R.P. et al. 1982. The production of bacteriocin-like substances by the oral bacterium *streptococcus salivarius*. Arch. Oral Biology, Vol: 27, pages 151-157. (See whole document).	4-20
X	Ross, K.F. et al. 1993. Isolation and characterization of the lantibiotic salivarcin A and its structural gene salA from *streptococcus salivarius* 20P3. Applied and Environmental Microbiology, Vol: 59, No: 7, pages 2014-21. (See abstract and pages 2014-2015).	4-20
X	Simpson, W.J. et al. 1995. A lantibiotic gene family widely distributed in streptoococcus salivarius and streptococcus pyogenes. Dev. Biol. Stand. Basel, Karger, Vol: 85, pages 639-643. (See whole document).	4-20
X	Huskins, W. C. et al. 1989. Inhibitory substances produced by *streptococcus salivarius* and colonization of the upper respiratory tract with group A streptococci. Epiderm. Inf., Vol: 102, pages 401-412. (See abstract and pages 401-402 and 404).	4-20
A	Darling, C.L. 1976. Antagonistic action of *streptococcus salivarius and streptococcus faecalis* to *mycobacterium tuberculosis*. Journal of Clinical Microbiology, Vol: 4, No: 4, pages 375-378. (See whole document).	1-26
A	WO 2003/070919 A1. (BLIS Technologies LTD). 28 August 2003. (See whole document).	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ2004/000025

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	3925160						
WO	0127143	AU	79737/00	BR	0014740	CA	2363713
		EP	1169340	NO	20013905		
WO	03070919						

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX